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# The inhalation effects of by-products from chlorination of heated indoor swimming pools on spinal development in pup mice

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ABSTRACT

*Introduction:* It has been postulated that swimming in heated indoor swimming pools in the first year of life is associated with the development of spinal deformity in children. We explored in pup mice whether exposure to certain disinfection by-products resulting from chlorination of heated pools would affect the future development of the spinal column.

*Methods:* Mice, from birth and for 28 consecutive days, were exposed to chemicals known to be created by disinfection by-products of indoor heated swimming pools. The study made use of a body fluid analogue and a chlorine source to recreate the conditions found in municipal pools. A cohort of 51 wild-type C57B6 mice, male and female, were divided into two groups: experimental (n = 29) and controls (n = 22). 24 mice were observed for 8 months (32 weeks), with 27 culled at 4 months (16 weeks). Serial CT scanning was used to assess the spines.

*Results:* Exposure to disinfection by-products resulted in an increase in the normal thoracic kyphotic spinal angle of the mice when compared with their controls at 10 weeks; experimental mice kyphosis range 35–82° versus 29–38° in controls. At 14 weeks the kyphosis of the experimental mice had reduced in size but never to that of the control group.

*Conclusion:* We have demonstrated the ability to influence spinal development in pup mice through environmental factors and shown that the developmental deformity became evident only after a significant latent period.

## 1. Introduction

Postnatal development constitutes a vulnerable period in a human's early infant life with regard to the effects of environmental hazards. Subtle effects during development, when the central nervous system (CNS) is both developing and maturing, can lead to functional deficits and increased risk of disease later in life. Barouki et al. (2012) stated that changes can be dependent on cell, tissue type and sex, and may not be apparent until after a latent period, which can last from months to years or decades. The White Paper of 2012 (Srader-Frechette, 2012) stated that children in their early postnatal developmental periods are particularly sensitive to developmental disruption by environmental-chemical exposures, with potentially adverse consequences for health later in life. An environmental assault can influence chemical marks on the DNA and the proteins that make up the genome, thus altering the genes without changing the DNA sequence (Dolinoy and Jirtle, 2008).

Normal growth of the spine can deform on the sagittal and/or coronal planes. McMaster et al. (2006) found in a case-control study a statistically significant association between the introduction of infants to heated indoor swimming pools and the subsequent development of an adolescent idiopathic scoliosis, which is a spinal deformity in the sagittal plane observed at the onset of the skeletal growth spurt in the early teenage years. A neurogenic hypothesis (McMaster, 2011) was proposed for the etiology of the deformity of adolescent idiopathic

*List of abbreviations:* BFA, Body fluid analogue; CNCl, Cyanogen chloride; CNS, Central nervous system; CSF, Cerebrospinal fluid; CT, Computerized tomography; DPD, N,N-diethyl-p-phenylenediamine; LPS, Lipopolysaccharide; $NCl_3$, Nitrogen trichloride; THM, Trihalomethane

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scoliosis which postulated that toxins from chlorine in such pools might cross the infant's blood-brain barrier and act deleteriously on the immature CNS, comprising brain and spinal cord.

The primary focus of the study was to assess possible effect of exposure to three chemical disinfection by-products (gases) found in heated indoor swimming pools: nitrogen trichloride ($NCl_3$), cyanogen chloride (CNCl), and chloroform.

The putative portals of entry into the blood for the chlorine-based chemicals could be dermal, oral, or respiratory. We looked at entry via the respiratory route. Entry of circulating small molecules to the brain would be via the blood-brain barrier, blood-cerebrospinal fluid (CSF) barrier, and the circumventricular organs. Karrow's review (Karrow, 2006) reported that inflammatory stressing during neonatal development, when critical windows of early-life are developing, can alter the programming of the neuroendocrine-immune axis. He states that there is some evidence to suggest that lipopolysaccharide (LPS) may be able indirectly to activate the hypothalamus-pituitary-adrenal axis by binding to LPS receptors on cells within the circumventricular organs and the blood-brain barrier.

During the first 6 months of human life CSF contains higher concentrations of proteins that are immunologically identical to proteins in plasma; this is attributed to mechanisms continued from fetal brain development rather than immaturity (Dzueguekewsja and Saunders, 1988). The clinical significance of this vulnerability might be that a large proportion of toxins which bind to plasma proteins can cross the choroid plexus (Saunders and Dziegielewska, 1998) and diffuse into the CSF, from where they can reach the individual brain structures. There is evidence of such apparent vulnerability of the blood-brain barrier which can occur in developing rodents' brains, particularly given differences in the fragility of cerebral blood vessels and abundance of transport proteins leading to increased permeability (Saunders et al., 2012).

Swimming pools in the UK safeguard public health against horizontal transmission of pathogens, usually by the use of calcium hypochlorite or sodium hypochlorite; however, these same agents may inadvertently be damaging human health. These oxidizers react with microorganisms, endogenous human excretions, and cosmetic chemicals to form a range of disinfection by-products (Kim et al., 2002). This study is concerned with three gaseous compounds found within chlorinated swimming pools: $NCl_3$, chloroform, and CNCl.

$NCl_3$ (trichloramine), considered to be the chlorine odor found in swimming pools, has been reported to be an irritant to the upper airways and eyes, with similar irritant potency to chlorine gas (Gagnaire et al., 1994). It is also found to increase hyperpermeability of lung epithelium (Bernard et al., 2006) and thus may weaken the protective nature of the lungs. Chloroform is grouped as a trihalomethane (THM) and known to be a common disinfection by-product. THMs are toxic to humans with repeated exposure and also known to be carcinogenic in rodents (Beddowes et al., 2003). The main exposure method and entry into the body is by inhalation (Aggazzotti, 1993). Finally, CNCl is present in all chlorinated swimming pools (Weaver et al., 2009) and is known to form when chlorine reacts with nitrogenous human excretion compounds such as urine (Weng et al., 2012) and is a registered chemical warfare agent.

The acceptable concentrations of CNCl, chloroform, and $NCl_3$ are not regulated for the swimming pool industry in the UK. Although there are work exposure limits for CNCl and chloroform, it is not standard practice for routine analysis because of impracticability. Numerous studies have been carried out on adults with regards to disinfection by-products (Gagnaire et al., 1994; Aggazzotti, 1993; Zwiener et al., 2007).

## 2. Materials and methods

### 2.1. Animals

All in-vivo experimental procedures were approved by the University of Edinburgh animal ethics committee and performed in compliance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986. 51 wild-type C57BL/6 mice, male and female, were divided into an experimental group and a control group. Animals came from multiple litters, and were randomly assigned to cage shortly after weaning at 19 days, stratified by sex. The experimental group consisted of 29 animals exposed to disinfection by-products (22 females and seven males). The control group had 22 animals (19 females and three males) that were not treated. We included substantially fewer males in our study, given that adolescent idiopathic scoliosis in humans is predominantly found in females. Animals were housed in unisex cages, with between two and five animals per cage. Control and exposed animals were housed separately. At 6 weeks siblings require separation to present reproduction.

Using an exposure chamber, pup mice together with the dam were placed into the chamber for 2 h a day from birth for 28 consecutive days. Once the gas exposure period was terminated, all animals were kept in ordinary cages (see below) and were left to develop, free from disinfection by-products. Having reached puberty 27 were culled at the age of 4 months (16 weeks). The remaining 24 animals (experimental 15, controls 9) were kept and CT scanned monthly up to the age of 7.5 months (30 weeks), which is equivalent to middle age in humans.

The animals in this study were obtained from in-house breeding colonies. The mice were maintained in polycarbonate boxes (335 $cm^2$ floor area) with weekly change of bedding of sterilized white Aspen shavings under conditions of 12 h of light and 12 h of darkness, with ambient temperature of 21 °C ( ± 2 °C) and humidity 55% ( ± 10%). Mice were given tap water triple filtered and an RMI standard diet of autoclaved pellets (22.4% protein, 4.2% fat, 1.15% calcium, 0.82% phosphorous, trace mineral, and vitamin fortified).

### 2.2. Chemistry

#### 2.2.1. Chemicals

The concentrations used for this study were chosen by reviewing literature regarding concentrations found within pools around the world (Table 1). We also considered the maximum exposure limits set by the World Health Organization.

**Table 1**
Concentrations selected for study.

| | Literature review average/ existing values in pools (mg/ $m^3$; ppmV) | Literature review average/ existing values in pools (μg/ L; ppb) | Original work exposure limits | Study concentration aim (mg/$m^3$) | Study concentration achieved (mg/L) |
|---|---|---|---|---|---|
| Nitrogen trichloride (MW: 120.365 g/mol) | 0.50 | 102.0 | 0.5mgm$^{-3}$ (French ref value) | 0.5 | 0.102 |
| Chloroform (MW: 119.36 g/mol) | 0.565 | 115.78 | 9.9mgm$^{-3}$ / 8 h | 0.5 | 0.102 |
| Cyanogen chloride (MW: 61.46 g/mol) | 0.303 | 120.80 | 0.77mgm$^{-3}$ / 15 min | 0.2 | 0.199 |

MW = molecular weight. ppmV = parts per million per volume, measured as a gas. ppb = parts per billion, measured as a liquid.

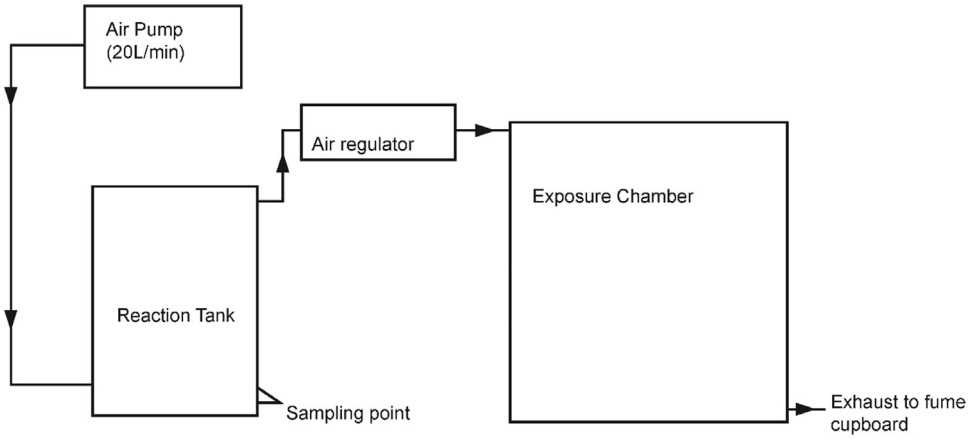



**Fig. 1.** The gaseous by-product delivery and exposure system.

**Table 2**
Body fluid analogue used in this study.

| | Substance name | Mass stock (g/L) |
|---|---|---|
| NH4Cl | Ammonium chloride | 2 |
| $CH_4N_2O$ | Urea | 14.8 |
| $C_4H_7N_3O$ | Creatinine | 1.8 |
| $C_6H_9N_3O_2$ | Histdine | 1.21 |
| $C_9H_9NO_3$ | Hippuric acid | 1.71 |
| $C_5H_4N_4O_3$ | Uric acid | 0.49 |
| $C_6H_8O_7$ | Cirtric acid | 0.64 |
| $C_6H_{14}N_4O_2$ | L Arginine | 1.35 |
| $C_2H_5NO_2$ | Glycine | 3.11 |

All chemicals were purchased from VWR and Merck Sigma Aldrich at Reagent grade or HPLC, where necessary. DPD1,3 reagents were purchased from DTK ltd. The exposure chamber was constructed by Easter Road Plastics ltd. from poly(methyl methacrylate) (acrylic).

### 2.2.2. Reaction tank and exposure chamber

Fig. 1 shows a schematic representation of the gaseous by-product delivery and exposure system. The physical set up of the experiment consisted of three reaction tanks of high density polyethylene (20 L), polyethylene pipe (6.35 mm diameter), rubber bungs, aquarium pump (20 L/min), three aquarium heaters (20 W), and three digital heaters. The system included a hygrometer to monitor humidity and an exposure chamber (120 L) to house the mice. The exposure chamber was situated within a fume cupboard. The full body exposure chamber was designed to house six standard international mouse cages, allowing for two dams and their litters per cage.

Three separate reaction tanks were used in a batch reaction process. A fresh 20 L container was created containing: body fluid analogue, pH buffer (phosphate buffer [$Na_2HPO_4$ (1 M) /$K_2HPO_4$ (1 M)] at pH 7 per tank allowing for a 0.1 M pH buffered solution), and sodium hypochlorite 3 days before each exposure session to allow for slow reaction times.

The target compounds were generated over a 72-h period in sealed 20 L containers and maintained at 26–27 °C. Once connected to the exposure chamber, air was supplied (4 L/min) to a submerged aquaria stone to simulate bather activity and allow for degassing. Samples were taken in 20 min intervals for headspace gas chromatography/mass spectrometry analysis, later the same day.

### 2.2.3. Exposure method

The mice specimen cages were introduced into the chamber each day at exactly the same time for a 2-h exposure period to the by-product gases. An air supply (4 L/min) passed through the reaction tank to the exposure chamber, allowing for degassing and therefore exposure of the by-product gases formed within the reaction tank to the pup mice. During every exposure period the following variables were measured and recorded every 40 min: free chlorine, total residual chlorine, combined chlorine, pH, reaction tank temperature, exposure tank temperature, and exposure tank relative humidity.

### 2.2.4. Body fluid analogue (BFA)

The dynamic of this system was to simulate an indoor heated swimming pool, temperature 26–27 °C, which becomes contaminated by human endogenous compounds from sweat, urine, etc, which would interact with primary disinfectants, such as hypochlorus acid/hypochlorite ion (chlorine). A body fluid analogue (BFA) was produced to simulate endogenous organic amino compounds, as previously described (Judd and Bullock, 2003). Table 2 shows the chemical components of the BFA.

The BFA mixture also included two amino acids: L-arginine and glycine. The modification ensured that the BFA mixture reflected the responsiveness of glycine to chlorine in producing CNCl, which is the most efficient CNCl precursor when chlorinating six organic nitrogen-containing compounds (Shang et al., 2000). In chlorination and UV exposure experiments, dichloromethylamine was found to be produced from creatinine, dichloroacetonitrile from L-histidine, CNCl from L-histidine, and $NCl_3$ from urea and L-arginine (Weng et al., 2012). Furthermore, chlorination of uric acid has been shown to produce CNCl and $NCl_3$ (Lian et al., 2014). With the aim of this experiment to produce both chloroform, $NCl_3$, and CNCl in one reaction tank, a balanced neutral pH of 7 was adopted so as not to favor either chemical constituent significantly. BFA was dosed as total organic carbon, which was calculated for the concentrated BFA measured to 7.19 g total organic carbon and an applied dose equaling that of 20 $mgL^{-1}$ in 20 L water reaction tank. A BFA solution in deionized water was produced bi-weekly.

### 2.2.5. Chlorine dosing

Active chlorine was sourced from sodium hypochlorite solution (12% active chlorine as $Cl_2$), and dosing in the 20 L reaction tank was equivalent to that as 100 $mgdm^{-1}$ $Cl_2$. Sodium hypochlorite solutions were kept refrigerated and out of light. The chlorine source was standardized every 2 days with N,N-diethyl-p-phenylenediamine (DPD) and photometer (Primelab). 100 ppm chlorine allowed for a sufficient chlorine supply to meet the chlorine demand of the system, leaving a portion of free chlorine for subsequent reactions. The chlorine dose was chosen to meet chlorine demand but not to exceed it, because it was found that the formation of CNCl is independent from free chlorine concentration and a higher concentration will promote CNCl hydrolysis (Na and Olson, 2006). Breakpoint chlorination is where a primary disinfectant is used to fully oxidize the ammonia-like species in the

water fully, to leave suitable residual free chlorine to allow for disinfection against cross contamination within the pool. This is practiced in municipal swimming pools around the world.

#### 2.2.6. Analytical standard production

CNCl standard was produced following the methods detailed by Wells (Wells, 1998). The method was adapted to produce standards starting with 1000 $\mu gL^{-1}$ and was subsequently diluted down for calibration standards. $NCl_3$ standards were produced based on methods from Shang and Blatchley (Shang and Blatchley, 1999). Chloroform and dichloroacetonitrile standards were purchased from Merck Sigma Aldrich. Dichloromethylamine standards were produced based on methods by Cimetiere and De Laat (Cimetiere and De Laat, 2009).

#### 2.2.7. Headspace gas chromatography mass spectrometry (GC-MS)

Trace GC-MS-MS Quantum with Triplus headspace sampler was used to determine CNCl (molecular weight 63.45 g/mol), chloroform (119.38 g/mol), dichloroactonitrile (109.94 g/mol), and dichloromethylamine (99.95 g/mol). Sampling was done by Triplus autosampler with a programmable temperature vaporizer injector method. Samples were taken (1 mL syringe draw), heated (70 °C), and incubated (4 min) with a syringe temperature of 120 °C. Sampling of the gases was taken by a liquid sample (5 mL) via a gate tap at the bottom of the reaction tank, directly into a headspace vial where it was immediately crimped, refrigerated, and analyzed within the same day.

#### 2.2.8. $NCl_3$ analysis

$NCl_3$ was analyzed by an air sampling method detailed by Hery et al. (1995). Sampling cassettes (SKG ltd.) were produced containing a PTFE (SKC ltd.) pre-filter, to remove any water droplets and filter mono/dichloramine followed by two quartz filters (37 mm; SKC ltd.) impregnated with diarsenic trioxide, glycerol, and sodium carbonate. Sampling cassettes were run with a portable air pump (Universal SKC Pump) operating inside the exposure chamber (1 $dm^3$ $min^{-1}$ for 120 min). The analysis method and equipment were sourced from Syclope Electronique. Filters were analyzed via chloride analysis with a ferric thiocyanate photometric method supplied by the company. $NCl_3$ in air was sampled within the exposure chamber for a 120-min exposure period each day. The sampling pump was running at 1 L/min for 120 min total. Each sampling cassette filter was analyzed twice.

### 2.3. Rotarod measurements

The rotarod is a test used to examine motor coordination, balance, fatigue and nerve damage in rodents. It consists of a rotating rod 5 cm in diameter, which is 15 cm above a platform. The rod rotates at whichever speed is selected and if a mouse falls off the rod, they depress a lever on the platform that stops the timer for that individual lane. There are several dividers creating lanes which allows each mouse to be tested without being influenced by others. Both study and experimental mice were tested on the rotarod on three separate occasions.

### 2.4. MRI

To detect changes in volume of different brain regions (eg, hypothalamus, pituitary, pineal gland), structural (T2-weighted) MRI scans were acquired covering the brain and upper spine in mice at 9, 21, and 28 days.

### 2.5. Computerized tomography (CT) scans in-vivo radiological imaging of mouse spine

Anesthesia was induced and maintained with isoflurane (2–2.5%, ratio of oxygen to nitrous oxide 50:50, 1 L/min) during the scanning procedure. Mice were then positioned head first in the scanner bed of a nanoPET/CT scanner (Mediso, Hungary) with the following settings: side or top view, X-ray energy of 50 kVp, exposure time of 300 ms, and maximum field of view. Any kyphotic deformity that was present was either visible or felt on palpation prior to the animal being anaesthetized. The animals were laid prone in the CT cradle to provide stability and present the animal rolling to either side.

The radiological method of observing any pathological changes in the vertebrae or intervertebral disc was by CT scanning, which was not subject to errors from positioning. Midline sagittal and frontal reconstructions of the CT scans were viewed. The standard technique for assessing spinal angulation, the Cobb method (Cobb, 1948), was used to assess spinal deformity. Angulation of the spine to either side in the coronal plane is termed a scoliosis, whereas a posterior convex angulation in the sagittal plane is termed a kyphosis.

Nucline software was used to make anatomical measurements and assess the magnitude of any possible spine deformity in the coronal and sagittal planes, according to the Cobb method (Cobb, 1948). The end vertebrae of the kyphosis were identified as those being maximally tilted into the concavity at the upper and lower ends of the kyphosis. The Cobb angle was formed by lines drawn along the top margin of the upper end vertebra and the lower margin of the lower end vertebra. These measurements were made digitally by a senior orthopedic spine deformity surgeon, blind to group and time and on multiple occasions at 4 week intervals. The intra-observer variability in the measurement of the Cobb angle for the senior surgeon was 5 degrees. A subset of animals (27: 14 experimental, 13 controls) had observations recorded at the 6-week old stage; all of these animals were then culled after the 14 weeks observation.

### 2.6. Statistical analysis

Data management from the CT scans was done manually using Excel 2016, and analyzed using Genstat 18th edition. The data were analyzed after application of a log transformation, excluding one outlier observation from a male control animal in week 30, on the grounds of fit. The data from week 6 were analyzed separately to that from the other weeks, since they had different statistical properties; this is appropriate given the immaturity of the adolescent skeleton at 6 weeks. Data from week 6 were analyzed using a linear mixed model, fitting litter and mouse as random effects, and sex and exposure as fixed effects. Data from weeks 10–30 were analyzed using a random coefficients regression model, allowing for random variability between mice. Cage use was also fitted as a random effect, and age (centered on the mean) was the regression explanatory variable. We estimated the effects of sex and exposure on the intercepts and slopes of the population regression models.

## 3. Results

Exposure to disinfection by-products caused statistically significant differences in the thoracic kyphotic spine at week 10. Fig. 2 shows the findings from the Cobb angle measurements in the experimental and control groups. A subset of animals were culled at 14 weeks (24 experimental, 13 controls) and are shown as 'Δ'. The remaining animals (15 experimental, nine controls) were culled at 30 weeks and are shown as 'O'.

When analysing individual cages over time, one study cage was significantly different when comparing results from week 22 to results of week 30 ($p = 0.0002$) as well as week 23 versus week 31 ($p = 0.0019$).

CT scanning of the mice showed no wedging or deformity of the vertebrae or end plate abnormalities. There could have been slight anterior wedging of each of the intervertebral discs within the kyphotic deformity, but this was so small at each level to make measurement impossible. No animal died as a result of the study, except for a dam whose pups were successfully fostered by an in-house dam and a 9-month-old mouse in our extended follow-up of 1 year. Although our

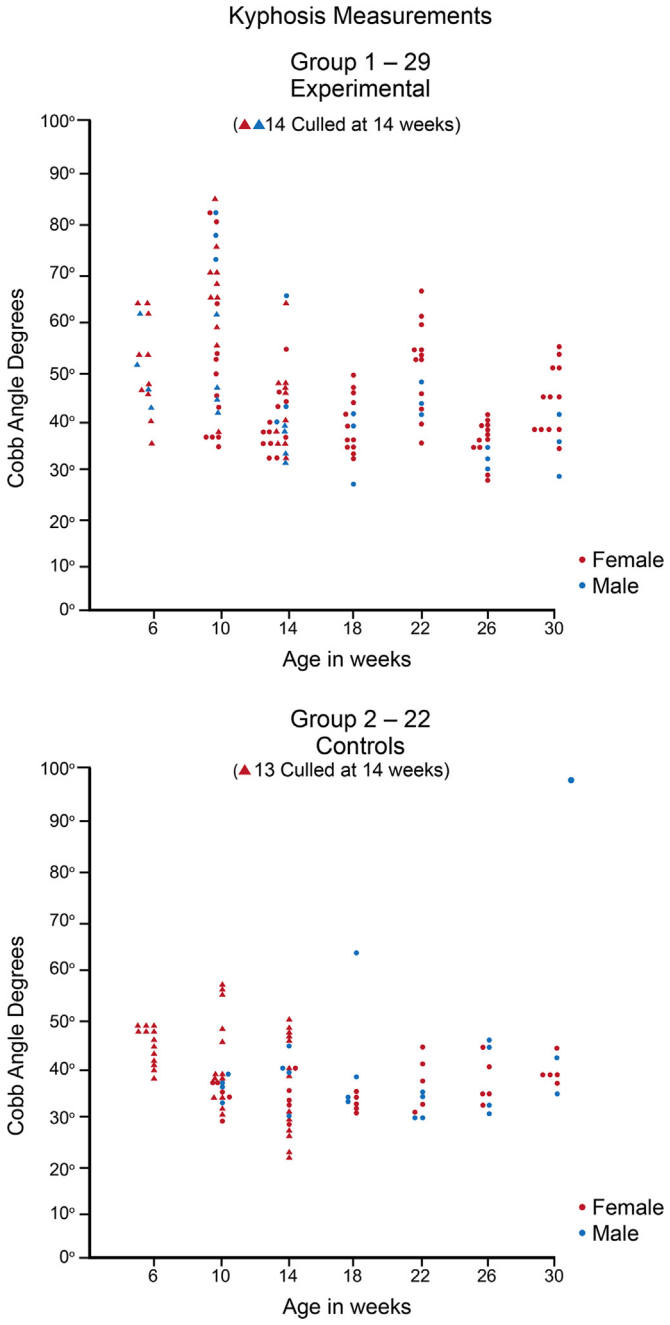



**Fig. 2.** Kyphosis measurements, from CT scans.

primary aim for this study was to observe the animals for an 8 month follow-up, they were CT scanned at 12 months before culling and it was noted that there were no macro changes such as tumors or abnormal organ size in any of the animals that could raise concern, and importantly no anatomical differences between the experimental and control animals. Hyper-intensity was observed in the male urethra for all animals, which could simply be urea crystals that tend to cement more in the male urethra than in the female urethra.

The concentrations of the three disinfection by-products varied greatly, with ranges of 0–198.58 μg/L for chloroform, 0–370 μg/L for CNCl, and 0.16–1.6 mg/m$^3$ for $NCl_3$. The average values for each target compound was approximately 64% compared with the concentration typically recorded in indoor heated swimming pools, with CNCl, chloroform, and $NCl_3$ achieving 48%, 55%, and 88% of the respective reported concentrations.

There were no differences between control females' and experimental females' body weights. As expected, the males were slightly heavier than the corresponding age-matched females. There were no control males to compare this parameter between age-matched male (control versus experimental) groups.

On culling at 16 weeks of age all animals were in good body condition. There were no differences in gross appearances of any organs when comparing control with experimental animals.

Fig. 3 shows the thoracic kyphotic angle of male animals at each point in time. As expected, there was no significant difference between the males and females in the control group. The variability of the kyphotic angle of the experimental animals of both sexes was significantly greater than that of the control group: for animals culled 30 weeks the mean was 11° (standard deviation [SD] 5.2°) for the experimental animals and 3.9° (SD 1.4°) for the control animals ($p < 0.001$). For animals culled at 14 weeks the mean was 12.2° (SD 5.6°) compared with 7.9° (SD 4.6°) for the control animals ($p < 0.05$). The range of the curve measurements in an individual animal varied from 11.8° to 53.7° in the experimental animals to 5.9–16° for the control animals.

Fig. 4 shows a boxplot of Cobb angle measured in male and female mice in both the experimental and control groups. This demonstrates how the mean and variability of the whole group change over time. One key point to note is that the mean observed angle is tending to increase between weeks 6 and 10 in the experimental group.

At week 10, we found statistically significant evidence that the mean of log-transformed angles measured in exposed mice was higher than that for control mice ($p = 0.04$). Over weeks 10–30, there was statistically significant evidence of differences in trend associated with sex ($p = 0.028$) and exposure ($p = 0.022$), with male mice and exposed mice showing, on average, steeper declines in mean log angle than their peers.

Fig. 5 shows the estimated means and population mean regression lines, categorized by exposure status and sex. These data are consistent with unexposed animals having responses that change less relative to age, regardless of sex, and that there is a difference between male exposed animals which show a steep decline in Cobb angle with increased age, relative to female exposed animals in whom there was less decline. However, in the absence of a statistically significant three-way interaction, this interpretation should be treated with caution.

Fig. 6 shows examples of the spinal morphology of experimental and control mice at week 10 and week 30. At week 10 the Cobb angle was 82.0° for the experimental mice versus 33.6° for the control mice; at week 30, the Cobb angle was 35.6°versus 34.4°

Fig. 7 shows results from MRI brain scans at 9, 21, and 28 days. At this stage of pup development, the MRI scans were not able to show anything of note. Images acquired with longer TE to generate more contrast (not shown) did not show any differences.

### 3.1. Rotarod measurements

Measurements obtained at 23 and 31 weeks, using a two-tailed *t*-test, showed no significant difference between the control and study cages at either speed at any of the three time points.

## 4. Discussion

Our study of the effect from inhalation of the identified gases on pup mice has shown that the only physical abnormality found, compared to the controls, was a time dependent reaction to the environmental assault manifesting as an abnormal curvature of the thoracic spine in the sagittal plane (Fig. 4). The remainder of the skeleton was normal and there were no other abnormalities seen on the CT or MRI scans.

The normal anatomical configuration and growth of the spine in mice and humans are similar in that longitudinal growth of the vertebra occurs at the chondro-epiphyseal end plates at the upper and lower ends of the vertebral body (Bick and Copel, 1950). The normal spine remains straight in the frontal plane without any evidence of a lateral

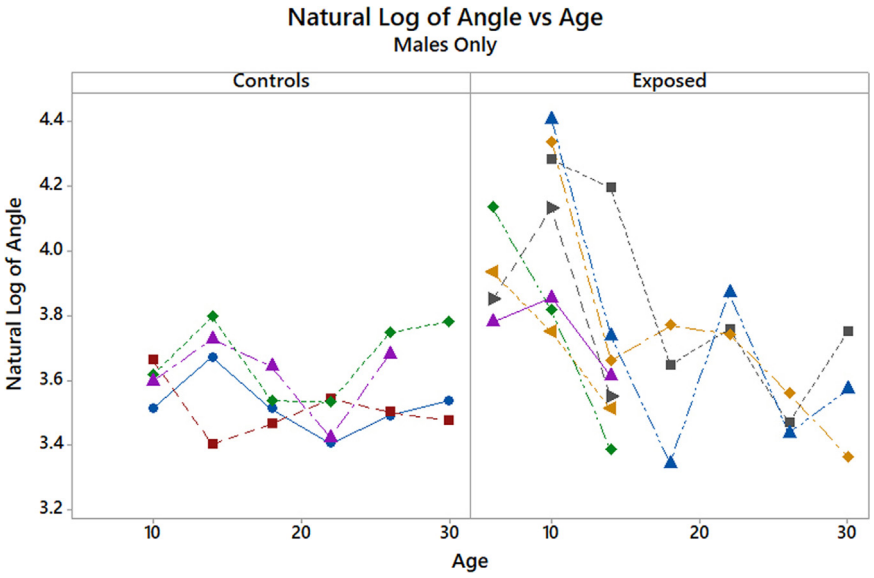



**Fig. 3.** Plot of angle against age for all male mice, control (left) and experimental (right).

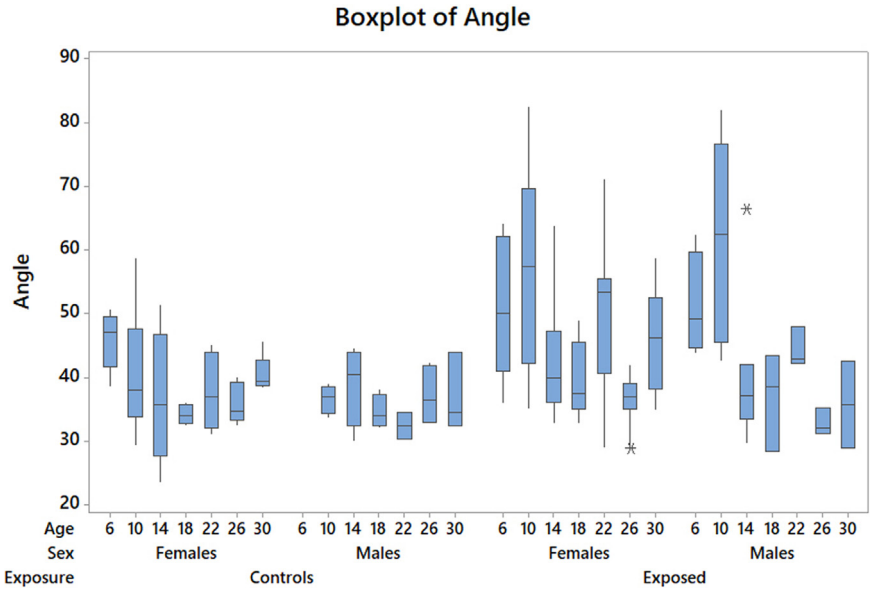



**Fig. 4.** Boxplots over time for different sub-categories.

curvature - i.e. scoliosis. However, in the sagittal plane when viewed from the side of both mice and humans there is a normal smooth posterior curvature (kyphosis) in the thoracic region. Burwell et al. (2008) have stated that gravitational force plays a role in the pathogeneses of deformity developing in the spinal column of humans. However, the spinal column of the quadruped is not subjected to gravity in its longitudinal axis and is therefore at less risk of developing a deformity in the frontal plane - i.e. scoliosis.

In comparative terms the period of maturity in mice which commences at 6 weeks can be equated to the onset of sexual maturity in adolescent humans. In our study, the newborn mice were exposed to the gases for 4 weeks after which there was a latency period before it was found that at 10 weeks, the exposed mice had developed an increase in the severity and variability of the normal thoracic kyphosis (range 35–82°) compared to the controls (range 29–38°) (Fig. 4). 4 weeks after the development of the hyperkyphosis there was a decrease in the severity of the deformity which continued over the ensuing 20 weeks (Fig. 4) but never to the levels found in the controls. At no stage did we observe any deformity in the frontal plane - i.e. scoliosis.

There are several explanations for the development of the hyperkyphotic deformity in pup mice. First, there could be a growth disturbance similar to that of Scheuermann's disease found in adolescent humans in which there is disordered endochondral ossification with irregularity of the vertebral growth plates associated with anterior wedging of the vertebrae. However, on CT scanning of the mice there was no wedging or deformity of the vertebrae or end plate abnormality to suggest a vertebral growth disturbance. There may have been slight anterior wedging at each of the discs within the kyphotic deformity but it is not possible to say whether this was a primary or secondary phenomenon. The large variation in sagittal Cobb angles of the hyperkyphosis suggests that the deformity was unstable in the exposed mice.

Secondly, there could be a neuropathic disturbance caused by the low molecular weight of the THMs crossing the blood-brain barrier and entering the brain via the CSF resulting in a neurological imbalance (Byczkowski et al., 2001). This could result in a developmental change occurring in the contour of the spinal column not as a scoliosis deformity but a kyphotic deformity in a quadruped. Dede et al. (2016), in a study using sagittal spinal CT scans of healthy children without

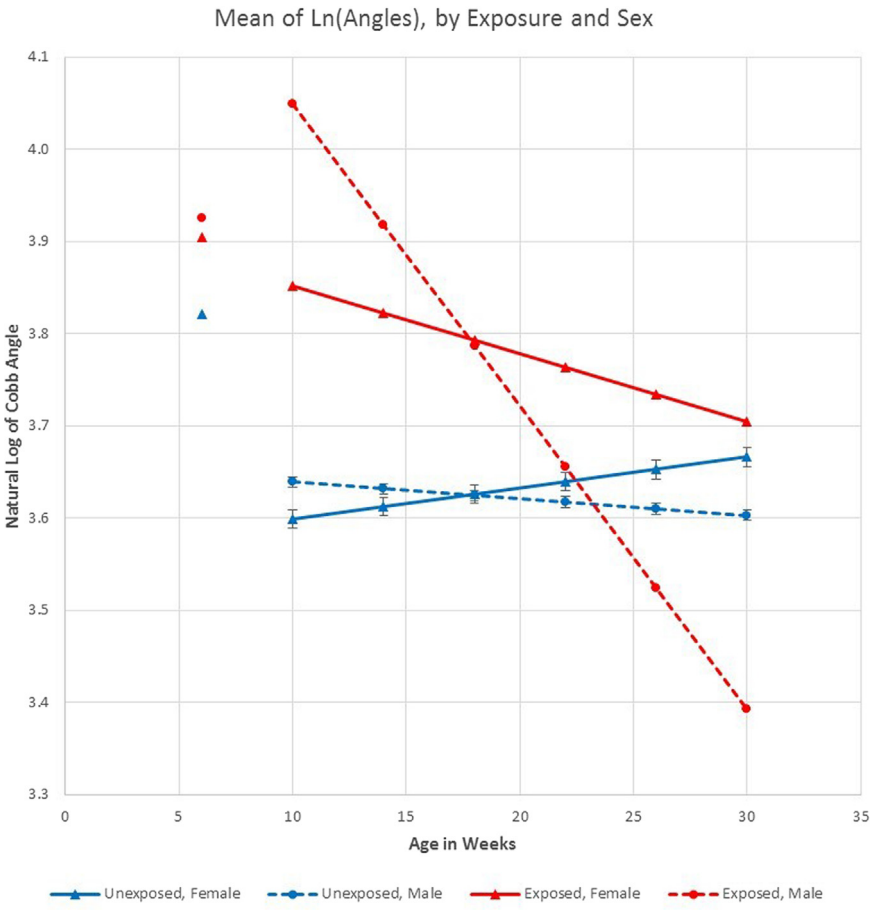



**Fig. 5.** Plot of the estimated means and population mean regression lines, by exposure status and sex.

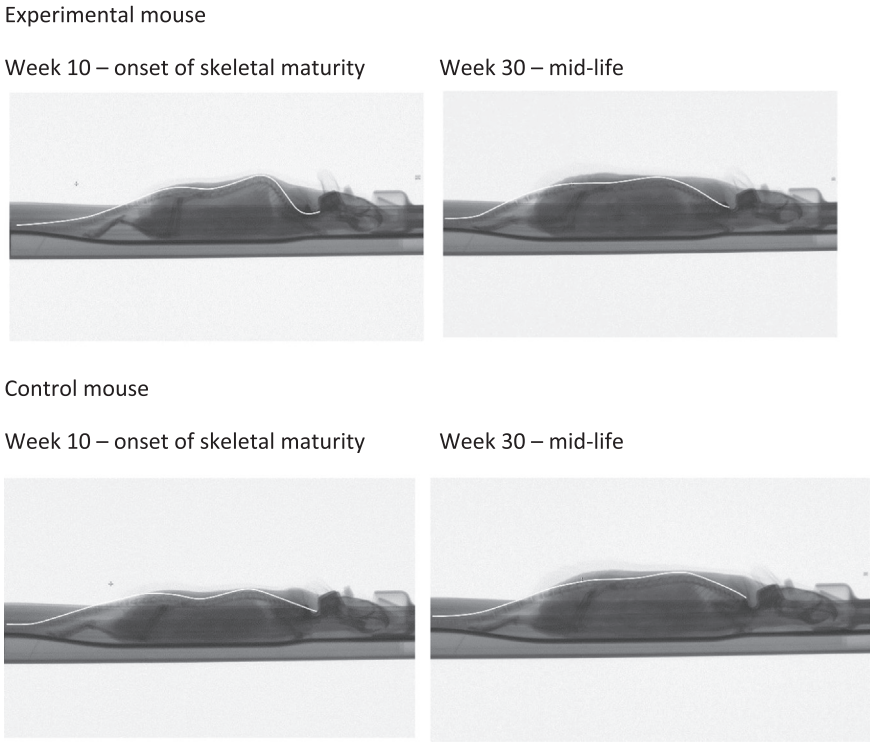
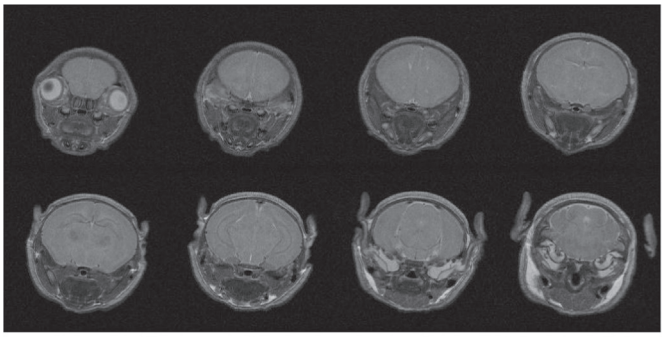
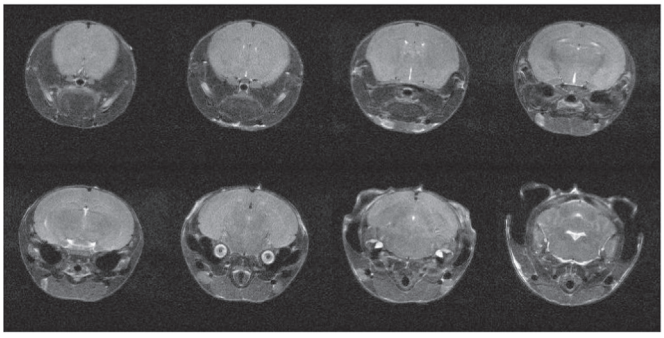


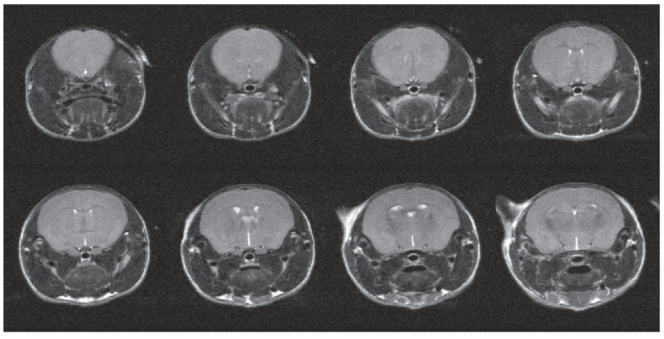

**Fig. 6.** Spinal morphology of experimental and control mice at weeks 10 and 30.



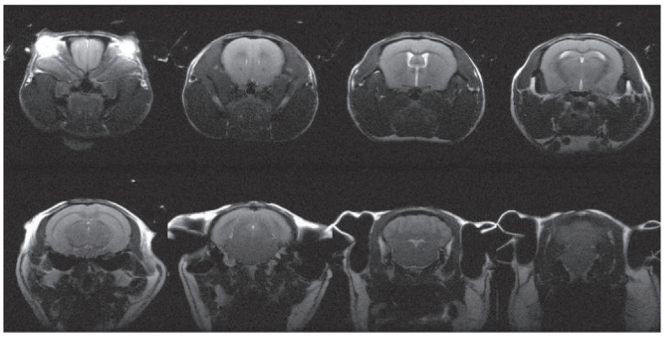
One animal, multiple slices, 9 days



One animal, multiple slices, 21 days



One animal, multiple slices, 28 days



Adult mouse from different study (note: images acquired with different RF coil).

**Fig. 7.** Results from MRI scans.

structural vertebral abnormalities, speculated that the development of the sagittal alignment and normal thoracic kyphosis was controlled by neurological balance rather than the individual shape of the vertebrae.

In our study the MRI scans of the mice brains between 9 and 28 days did not show any gross abnormalities (Fig. 7). In addition, the rotarod assessment at 23 and 31 weeks did not demonstrate a change in neuromotor coordination. Thus there were no objective neurological abnormalities observed in our animal model.

Our results showed that the maximum spinal deformity occurred at week 10. The statistically significant differences over weeks 10–30 during which the hyperkyphosis partly resolves can be interpreted as showing that there was a partial recovery from the environmental insult in the experimental male mice and to a lesser degree in the female mice. In an adolescent human with a kyphotic deformity there is a tendency to deteriorate during skeletal growth. This may be due to the unique mechanics of the human's fully upright biomechanical spinal loading, in which gravity has been acknowledged as playing a part in curve progression. This does not apply to the quadruped in the same way.

Countries such as Germany have recognized the potential hazardous environment from the chlorination of heated indoor swimming pools, primarily for respiratory and dermatological reasons (UBA recommendation and DIN standards, 2015). However, the data presented here suggest that there could be an added effect on the future development of the spinal column in infants.

## 5. Conclusion

In this study we report for the first time that exposure of pup mice in the first 4 weeks of life to identifiable gases found in heated indoor swimming pools is associated with the development of a hyperkyphosis in the sagittal plane of the spine. Following exposure to the gases there was a latency period of 6 weeks before the deformity developed. This raises the possibility that early exposure of infants to heated indoor swimming pools may have a later effect on subsequent spinal development.

## Authors' contributions

MEMcM is the principal researcher. AHRWS is the HO animal license holder and co-author. CA-S is responsible for the Chemistry section; AADST and CAC for the X-ray and CT section; MAJ for the MRI section; KMcG for the Neuromuscular testing section; and DMcN for animal husbandry. Each author drafted part of the first version of the manuscript and each contributed to the revision. All authors read and approved the final manuscript.

## Declarations

### *Ethics approval*

All in-vivo experimental procedures were approved by the University of Edinburgh animal ethics committee and performed in compliance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986.

## Competing interests

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest.

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## References


Aggazzotti, G., 1993. Chloroform in alveolar air of individuals attending indoor swimming pools. Arch. Environ. Health 48, 250–254.

Barouki, R., Gluckman, P.D., Grandjean, P., Hanson, M., Heindel, J.J., 2012. Developmental origins of non-communicable disease: implications for research and public health. Environ. Health 11, 42.

Beddowes, E.J., Faux, S.P., Chipman, J.K., 2003. Chloroform, carbon tetrachloride and glutathione depletion induce secondary genotoxicity in liver cells via oxidative stress. Toxicology 187, 101–115.

Bernard, A., Carbonnelle, S., de Burbure, C., Michel, O., Nickmilder, M., 2006. Chlorinated pool attendance, atopy, and the risk of asthma during childhood. Environ. Health Perspect. 114, 1567–1573.

Bick, E.M., Copel, J.W., 1950. Longitudinal growth of the human vertebra. A contribution to human osteogeny. J. Bone Jt. Surg. 32-A, 803–814.

Burwell, R.G., Dangerfield, P.H., Freeman, B.J.C., 2008. Concepts on the pathogenesis of adolescent idiopathic scoliosis. Bone growth and mass, vertebral column, spinal cord, brain, skull, extra-spinal left-right skeletal length asymmetries, disproportions and molecular pathogenesis. Stud. Health Technol. Inform. 135, 3–52.

Byczkowski, J.Z., Kacew, S., Miller, R.K., Pelkonen, O., Saunders, N., 2001. Exploration of perinatal pharmacokinetic issues. final report (P.C-13. May 10). Prep. US Environ. Prot. Agency (P.C-13. May 10).

Cimetiere, N., De Laat, J., 2009. Henry's law constant of N,N-dichloromethylamine: application to the contamination of the atmosphere of indoor swimming pools. Chemosphere 77, 465–470.

Cobb, J.R., 1948. Outline for the study of scoliosis. Am. Acad. Orthop. Surg. Instr. Course Lect. 5, 261–275.

Dede, O., Büyükdogan, K., Demirkıran, H.G., Akpınar, E., Yazıcı, M., 2016. The development of thoracic vertebral sagittal morphology during childhood. Spine Deform. 4, 391–394.

Dolinoy, D.C., Jirtle, R.L., 2008. Environmental epigenomics in human health and disease. Environ. Mol. Mutagen. 49, 4–8.

Dzueguekewsja, K.M., Saunders, N.R., 1988. The development of the blood-brain barrier: proteins in fetal and neonatal CSF, their nature and origins. In: Meisami, E., Timiras, P.S. (Eds.), Handbook of human growth and development biology. Volume 1. Neural, sensory, motor and integrative development. CRC Press, Boca Raton, pp. 169–191.

Gagnaire, F., Azim, S., Bonnet, P., Hecht, G., Hery, M., 1994. Comparison of the sensory irritation response in mice to chlorine and nitrogen trichloride. J. Appl. Toxicol. 14, 405–409.

Hery, M., Hecht, G., Gerber, J.M., Gender, J.C., Hubert, G., Rebuffaud, J., 1995. Exposure to chloramines in the atmosphere of indoor swimming pools. Ann. Occup. Hyg. 39, 427–439.

Judd, S.J., Bullock, G., 2003. The fate of chlorine and organic materials in swimming pools. Chemosphere 51, 869–879.

Karrow, N.A., 2006. Activation of the hypothalamic-pituitary-adrenal axis and autonomic nervous system during inflammation and altered programming of the neuroendocrine-immune axis during fetal and neonatal development. Lessons learned from the model inflammagen, lipopolysaccharide. Brain Behav. Immun. 20, 144–158.

Kim, H., Shim, J., Lee, S., 2002. Formation of disinfection by-products in chlorinated swimming pool water. Chemosphere 46, 123–130.

Lian, L., E.Y., Li, J., Blatchley III, E.R., 2014. Volatile disinfection byproducts resulting from chlorination of uric acid: implications for swimming pools. Environ. Sci. Technol. 48, 3210–3217.

McMaster, M.E., 2011. Heated indoor swimming pools, infants and the pathogenesis of adolescent idiopathic scoliosis: a neurogenic hypothesis. Environ. Health 10, 86.

McMaster, M.E., Lee, A.J., Burwell, R.G., 2006. Physical activities of patients with adolescent idiopathic scoliosis (AIS) compared with a control group: implications for etiology and possible prevention. J. Bone Jt. Surg. (Br.). 88-B (Suppl II), 225.

Na, C., Olson, T.M., 2006. Mechanism and kinetics of cyanogen chloride formation from the chlorination of glycine. Environ. Sci. Technol. 40, 1469–1477.

Saunders, N.R., Dziegielewska, K.M., 1998. Transport in the developing brain. In: Pardridge, W.M. (Ed.), An introduction to the blood-brain barrier. Cambridge University Press, Cambridge, pp. 277–288.

Saunders, N.R., Liddelow, S.A., Dziegielewska, K.M., 2012. Barrier mechanisms in the developing brain. Front Pharmacol. 3, 1–18.

Shang, C., Blatchley III, E.R., 1999. Differentiation and quantification of free chlorine and inorganic chloramines in aqueous solution by MIMS. Environ. Sci. Technol. 33, 2218–2223.

Shang, C., Woei-Long, G., Blatchley III, E.R., 2000. Breakpoint chemistry and volatile byproduct formation resulting from chlorination of model organic-N compounds. Environ. Sci. Technol. 34, 1721–1728.

Srader-Frechette, K., 2012. Taking action on developmental toxicity: scientists' duties to protect children. Environ. Health 11, 61.

UBA recommendation and DIN standards, 2015. 19643 part 5; November.

Weaver, W.A., Li, J., Wen, Y., Johnston, J., Blatchley, M.R., Blatchley 3rd, E.R., 2009. Volatile disinfection by-product analysis from chlorinated indoor swimming pools. Water Res. 43, 3308–3318.

Wells, W., 1998. An in situ synthesis of cyanogen chloride as a safe and economical aqueous standard. Water Res. 32, 2865–2869.

Weng, S., Li, J., Blatchley 3rd, E.R., 2012. Effects of UV 254 irradiation on residual chlorine and DBPs in chlorination of model organic-N precursors in swimming pools. Water Res. 46, 2674–2682.

Zwiener, C., Richardson, S.D., DeMarini, D.M., Grummt, T., Glauner, T., Frimmel, F.H., 2007. Drowning in disinfection byproducts? Assessing swimming pool water. Environ. Sci. Technol. 41, 363–372.